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Human Immunodeficiency Virus Type 1 Vif Protein Is Packaged into the Nucleoprotein Complex through an Interaction with Viral Genomic RNA

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The human immunodeficiency virus type 1 (HIV-1) Vif protein plays a critical role in the production of infectious virions. Previous studies have demonstrated the presence of small amounts of Vif in virus particles. However, Vif packaging was assumed to be nonspecific, and its functional significance has been questioned. We now report that packaging of Vif is dependent on the packaging of viral genomic RNA in both permissive and restrictive HIV-1 target cells. Mutations in the nucleocapsid zinc finger domains that abrogate packaging of viral genomic RNA abolished packaging of Vif. Additionally, an RNA packaging-defective virus exhibited significantly reduced packaging of Vif. Finally, deletion of a putative RNA-interacting domain in Vif abolished packaging of Vif into virions. Virion-associated Vif was resistant to detergent extraction and copurified with components of the viral nucleoprotein complex and functional reverse transcription complexes. Thus, Vif is specifically packaged into virions as a component of the viral nucleoprotein complex. Our data suggest that the specific association of Vif with the viral nucleoprotein complex might be functionally significant and could be a critical requirement for infectivity of viruses produced from restrictive host cells.

The human immunodeficiency virus type 1 (HIV-1) Vif protein plays an important role in regulating virus infectivity (20, 62). The lack of a functional Vif protein results in the production of virions with reduced or abolished infectivity (20, 35, 62). This effect of Vif on virus infectivity is producer cell dependent and can vary by several orders of magnitude (2, 6, 7, 19, 20, 22, 35, 51, 62, 66). Virus replication in nonpermissive cell types such as H9 is strictly dependent on Vif, while Vif-defective viruses can replicate efficiently in permissive hosts such as Jurkat cells. The cellular factors determining the requirement for Vif are currently not known. Results from heterokaryon analyses which involved the fusion of restrictive with permissive cell types suggest the presence of an inhibitory factor in restrictive cell types (41, 54). However, the identity of the proposed inhibitory factor and its mode of action remain elusive. Recent work investigating the ability of Vif from different lentiviruses for cross-species transcomplementation suggests that Vif itself functions in a host cell-dependent manner, supporting the notion that Vif may interact with as yet unknown cellular factors (57).

Although *vif* genes are present in all lentiviruses with the exception of equine infectious anemia virus (44), there is relatively little sequence conservation between different Vif variants. Nevertheless, HIV-1 Vif was found to be capable of functionally complementing Vif-defective HIV-2 and simian immunodeficiency virus strain mac (SIV_{mac}) isolates (48, 57, 58), suggesting common functional domains and a common mode of action. Similarly, HIV-2 Vif was capable of complementing HIV-1 Vif defects (48, 57).

Vif is a basic, 23-kDa protein that is expressed from a singly spliced mRNA in HIV-infected cells. Immunocytochemical analyses reveal a largely cytoplasmic localization of Vif (24, 34, 53). Two recent reports suggest that Vif associates with viral genomic RNA in vivo and in vitro (15, 72), and deletions in the N-terminal and central regions of Vif were found to affect its ability to bind to poly(G)-conjugated agarose beads in vitro (72). Aside from its affinity to RNA, Vif was reported to associate with cellular membranes through a mechanism involving a basic C-terminal domain in Vif (24, 26, 60). This same domain was also reported to be responsible for the interaction of Vif with the Gag precursor $Pr55^{gag}$ (8), and mutations in the basic domain were found to abolish biological activity of Vif (8, 26). In addition to the C-terminal basic domain, Vif proteins contain two conserved cysteine residues which are important for its biological activity (10, 40). The precise function of these cysteine residues in unclear; however, they do not appear to be involved in the formation of intramolecular disulfide bridges and are more likely to constitute part of a functional domain in Vif (60). Finally, a significant amount of Vif can be found in association with the intermediate filament network in virus-producing cells (34); however, the domain(s) in Vif responsible for this association remains unclear.

Despite the severe impact of Vif defects on virus infectivity, its mechanism of action has thus far remained obscure. It is generally accepted that Vif-deficient viruses can attach to and penetrate host cells but are blocked at a postpenetration step early in the infection cycle (3, 11, 13, 48, 55, 66). Yet comparison of virion morphology or protein composition between

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wild-type and Vif-defective virions has thus far been inconclusive and produced conflicting results (7, 9, 21, 32, 45, 52). Several reports have suggested that Vif affects the stability of the viral nucleoprotein complex (32, 46, 55). In particular, NC and reverse transcriptase were found to be less stably associated with viral cores in the absence of Vif (46). Nevertheless, Vif is generally believed to function within the virus-producing cell. This assumption is largely based on the observation that relatively small amounts of Vif seem to be packaged, with estimates ranging from less than 1 to 100 molecules of Vif per virion (10, 16, 21, 39). Furthermore, packaging of Vif into virus particles is generally believed to be nonspecific, leading to questions as to the functional significance of Vif incorporation into virions (10, 16, 56).

In the current study, we performed an in-depth biochemical analysis of Vif in purified virions from permissive and restrictive host cells to investigate the specificity of Vif incorporation into virions. Pulse-chase analysis of single-cycle-infected H9 cells did not reveal any Vif-dependent differences in viral protein processing and maturation consistent with recent reports by other investigators (15, 21, 45). Instead, detergent extraction of purified virions demonstration an association of Vif with the nucleoprotein complex. Interestingly, HIV-1 variants carrying mutations in the nucleocapsid zinc finger domains abolished Vif packaging. In addition, an RNA-packaging defective virus was significantly impaired in packaging of Vif. Finally, deletion of a putative RNA-binding motif between residues 75 and 114 in Vif abolished its packaging into virions. Taken together, our results indicate that virion packaging of Vif is specific and is mediated by interactions with the viral genomic RNA.

MATERIALS AND METHODS

Plasmids. The full-length molecular clone pNL4-3 (1) was used for the production of wild-type infectious virus. An Env-defective variant, pNLenv-1, was constructed by deleting a *Kpn*I-*Bgl*II fragment from the pNL4-3 *env* gene (nucleotides 6343 to 7611 in the pNL4-3 sequence). Two RNA packaging-defective nucleocapsid mutants of NL4-3, pRB73-B, carrying histidine-to-cysteine mutations in the nucleocapsid zinc finger domains (H23C and H44C), and pDB653, carrying cysteine-to-serine mutations (C15S, C18S, C28S, C36S, C39S, and C49S), have been described elsewhere (27, 30). Based on Northern blot analysis, the full-length genomic RNA content of DB653 virions is <10% that of wild-type virus (30). Similarly, the full-length genomic RNA content of RB73-B virions was reduced to approximately 5% that of wild-type virus (27). Another RNA packaging-defective virus, C-Help, was obtained from Hideki Mochizuki (42). C-Help is defective for packaging of viral genomic RNA due to a deletion of a putative RNA packaging signal. In addition, C-Help lacks the two viral long terminal repeats (LTRs) and carries a deletion in the *env* gene. Plasmid pHCMV-G contains the vesicular stomatitis, virus (VSV) glycoprotein G (VSV-G) gene expressed from the immediate-early gene promoter of human cytomegalovirus (69) and was used for the production of VSV-G pseudotypes. For transient expression of Vif, the subgenomic expression vector pNL-A1 (62) was employed. This plasmid expresses all HIV-1 proteins except for *gag* and *pol* products. A Vif-defective variant of pNL-A1, pNL-A1Δvif, was constructed by deletion of an *Nde*I-*Pfl*MI fragment in *vif*, resulting in a translational frameshift following amino acid 28 (34). The Vif deletion mutant VifΔG (deletion of amino acids 75 to 114) was created by two-step PCR amplification. The initial set of PCR fragments was produced using primers A5 (TTAGACCAGA TCTGAGCCTG GGAGC), A3 (TAGCAGAGTC TGAAAATGTA TGCAGACCCC), and B5 (TGGGGTCTGC ATACATTTTC AGACTCTGC) and B3 (AAACAGCAGT TGTTGCAGAA TTC). The resulting PCR products A and B were column purified, mixed at equimolar ratios, and used as templates for a second round of amplification using the flanking primers A5 and B3. The final PCR product was purified, digested with *Bss*HII and *Eco*RI, and cloned into the *Bss*HII and *Eco*RI sites of pNL-A1. The in-frame deletion mutants VifΔB (deletion of residues 157 to 184), VifΔC (deletion of residues 144 to 149), and VifΔD (deletion of residues 23 to 43) were constructed using similar two-step PCR approaches. The presence of the desired deletions and the absence of additional PCR-induced mutations were verified by sequence analysis.

The 8-amino-acid Flag epitope tag (DYKDDDDK) was added to the C terminus of Vif in pNL-A1 and pNL-A1vifΔB by PCR-based mutagenesis using the 5′ primer GTC AGG GAG TCT CCA TAG AAT GGA GGA AAA AGA G and the 3′ primer TTG CAG AAT TCT AGA TCA CTT GTC GTC ATC GTC TTT ATA ATC GTG TCC ATT CAT TGT GTG G for amplification of pNL-A1 and pNL-A1vifΔB template DNAs. The resulting PCR products were cleaved with *Pfl*MI and *Eco*RI and cloned into the *Pfl*MI and *Eco*RI sites of pNL-A1. The plasmids pNL-A1vifΔB and pNL-A1vifΔB_{Flag} carry a deletion in *vpr* which renders the *vpr* gene nonfunctional in these constructs. Full-length molecular clones of HIV-1 carrying the various deletions in Vif were constructed by cloning the corresponding fragments of pNL-AIvifΔB, pNL-AIvifΔB_{Flag}. pNL-AIvifΔD, or pNL-AIvifΔG into pNL4-3 as follows. The VifΔD and VifΔG deletion mutants were first subcloned into plasmid pSE1x (63) using the *Bsp*MI and *Eco*RI sites and in a second step cloned into pNL4-3 using *Age*I and *Eco*RI sites. VifΔB and VifΔB_{Flag} were cloned directly into pNL4-3 using the unique *Pfl*MI and *Eco*RI sites. The construction of pNL4-3Δvif was described before (34). As a control for the lack of Vpr function in VifΔB and VifΔB_{Flag} variants, a *vpr*-defective plasmid, pNL4-3Δvpr, was constructed by digestion of pNL4-3 with *Eco*RI, filling in of the site with *Escherichia coli* DNA polymerase I, and religation.

Antisera. Serum (APS) from an HIV-positive patient was used to detect HIV-1-specific proteins. The serum does not recognize Vif or Nef and reacts only poorly with gp120 in immunoblot assays. A polyclonal, monospecific antiserum to Vif was raised in rabbits against *E. coli*-derived fusion proteins (34) and used for all immunoprecipitation and immunoblotting analyses. Integrase-specific peptide antibodies were a gift from D. Grandgenett and were obtained through the National Institutes of Health AIDS Research and Reagent Program. A nucleocapsid-specific antibody was obtained from the AIDS Vaccine Program, Biological Products Laboratory, National Cancer Institute, Frederick, Md. (animal number 77, bleed 000900). Antibodies to gp41 were obtained from Fitzgerald Industries International, Inc. (Concord, Mass.). The monoclonal antibody M2, recognizing the Flag epitope, is a product of Eastman Kodak (New Haven, Conn.).

Tissue culture and transfections. HeLa cells were propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). H9, A3.01, and Jurkat T-cell lines were maintained in complete RPMI 1640 medium supplemented with 10% FBS. Peripheral blood mononuclear cells (PBMC) were treated with phytohemagglutinin (PHA; 1μg/ml) for 3 to 4 days in RPMI containing 10% FBS. Prior to infection, cells were washed once to remove PHA and transferred into RPMI–10% FBS containing interleukin-2 (20 U/ml).

For transfection of HeLa cells, cells were grown in 25-cm^2 flasks to about 80% confluency. Cells were transfected either by the calcium phosphate precipitation method as described elsewhere (34) or with Lipofectamine (Life Technologies, BRL) following the manufacturer's recommendations. For calcium phosphate transfection, a total of 20 to 30 μg of plasmid DNA was used per 25-cm^2 flask. For Lipofectamine transfections, a total of 4 to 5 μg of plasmid DNA per 25-cm^2 flask was used. Cells were harvested 48 h posttransfection.

Preparation of virus stocks. Virus stocks were prepared by transfecting HeLa cells with appropriate plasmid DNAs (5 μg/25-cm^2 flask) using Lipofectamine. For the production of virus stocks pseudotyped with the VSV glycoprotein G, HIV plasmids were cotransfected with pHCMV-G (4 μg of viral plasmid plus 1 μg of pHCMV-G per 25-cm^2 flask). Virus-containing supernatants were harvested 48 h after transfection. Cellular debris was removed by centrifugation (3 min, 3,000 × *g*), and clarified supernatants were filtered (0.45 μm) to remove residual cellular contaminants. Filtered supernatants were then concentrated by ultracentrifugation (3,5000 rpm, 1 h, SW41 rotor [Beckman]). Concentrated virions were suspended in RPMI medium and further purified by linear sucrose gradient centrifugation, by sucrose step-gradient analysis, or by pelleting through a 20% sucrose cushion.

Metabolic labeling, cell fractionation, and immunoprecipitation. Transfected HeLa cells (approx. 10×10^6) were metabolically labeled for 90 min with [^{35}S] methionine (2 mCi/ml; ICN Biomedical. Inc. Costa Mesa, Calif.). After the labeling, cells were washed once with phosphate-buffered saline (PBS) to remove free isotope and suspended in PBS. For some experiments, RNase A was added to the samples at this point (0.5 mg/ml final concentration). Cells were lysed by three cycles of freezing and thawing (3 min each at −70 and 37°C, respectively). Cells were vortexed for 5 s between each cycle. Insoluble material was pelleted for 3 min at 15,000 × *g*, and the supernatant (fraction 1) was collected. The pellet was extracted with CHAPS buffer, containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 100 mM NaCl, 0.5% (vol/vol) CHAPS (3-[(3-cholamidopropyl)-diethyl-

ammonio]-1-propanesulfonate) and 0.2% deoxycholate (DOC), incubated on ice for 5 min, vortexed, and pelleted as before. Detergent-soluble material present in the supernatant (fraction 2) was collected. Proteins present in the detergent-resistant pellet fraction (fraction 3) were solubilized by boiling in sample buffer (4% sodium dodecyl sulfate [SDS], 125 mM Tris-HCl [pH 6.8], 10% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue) for 10 min at 95°C. Prior to immunoprecipitation, all fractions were adjusted to equal volume, ionic strength, and detergent concentration. Cell lysates were precleared on Gamma-Bind Plus Sepharose beads (Pharmacia LKB Biotechnology, Piscataway, N.J.) followed by immunoprecipitation with appropriate antisera as indicated in the text. Proteins were solubilized by boiling in sample buffer and separated by SDS-polyacrylamide gel electrophoresis (PAGE). Radioactive bands were visualized by autoradiography, and quantitation was performed using a Fuji BAS 2000 Bio-Image analyzer. To determine total intracellular levels of Vif, whole-cell extracts were prepared in essence as described above except that the contents of the three fractions were pooled.

Linear sucrose gradient and step gradient analysis. Linear sucrose gradients for the purification of HIV virions were prepared as follows: 2.5 ml of 50 or 60% sucrose solutions were placed into SW55 centrifuge tubes at room temperature and overlaid with a 10% sucrose solution. The tubes were then sealed, and the gradient was established by rotating the tubes for 50 s at an angle of 86.0° and a speed of 25 rpm in a BioComp Gradient Master (BioComp Instruments, Inc., Fredericton, Canada). To load virus onto the gradients, 750 μl of the sucrose solution were removed from the top of the gradients and replaced with 500 μl of concentrated virus preparations. Gradients were centrifuged in an SW55Ti rotor for 75 min at 35,000 rpm at 4°C. Thirteen individual fractions (385 μl each) were collected manually from the top of the gradients.

Sucrose step gradients were prepared as follows: 2.0 ml of a 60% sucrose solution was placed into the bottom of SW55 centrifuge tubes and overlaid with 2.1 ml of a 20% sucrose solution. Immediately prior to addition of concentrated virus stocks (500 μl), the step gradients were overlaid with 100 μl of a protease inhibitor cocktail (Complete; Boehringer) and 100 μl of either PBS or 1% Triton X-100. Samples were then centrifuged in an SW55Ti rotor for 60 min at 35,000 rpm and 4°C. Three fractions of 1.1 ml each were collected from the top and combined with 100 μl of protease inhibitor cocktail each.

Aliquots of each fraction of linear gradients or step gradients were subsequently processed for reverse transcriptase analysis (RT assay) or immunoblotting.

Endogenous RT assay. The presence of viral genomic RNA in gradient fractions was analyzed by endogenous reverse transcription. This assay is based on the synthesis of tRNA-primed cDNA by the virion-associated reverse transcriptase. For the analysis of individual step gradient fractions, each fraction was adjusted to 50 mM Tris-HCl (pH 7.8), 75 mM KCl, 2 mM dithiothreitol, 5 mM $MgCl_2$, and 0.05% NP-40. Unlabeled dATP, dCTP, and dGTP were added to 330 μM each; [α-^{32}P]dTTP (10 mCi/ml) was added to 0.5 μM. To distinguish cDNA synthesis from genomic viral RNA and spliced mRNA, samples were spiked with a synthetic oligonucleotide (Z85) with the ability to direct the synthesis of minus-strand strong-stop cDNA from unspliced viral genomic RNA but not from spliced mRNA. The 18-based Z85 oligonucleotide has the sequence 5′-ACT GAC GCT CTC GCA CCC-3′ and is complementary to the NL4-3 sequence (positions 337 to 354 on the viral RNA). Samples were incubated at 37°C for 15 min and chased for 5 min with a complete deoxynucleoside triphosphate mix (dGTP, dATP, dCTP, and dTTP, all at 250 μM final concentration). Reactions were stopped by addition of EDTA (20 mM). Samples were extracted with buffer-saturated phenol, followed by extraction with chloroform and precipitation with ethanol. Pellets were washed with 70% ethanol and dried. Samples were then suspended in 10 μl of formamide buffer (80% formamide, 10% glycerol, 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, 0.02% bromophenol blue, 0.02% xylene cyanole blue) and denatured for 5 min at 95°C. Then 3 μl of each fraction was subjected to RNase treatment (2.5 mg of RNase A per ml, 60 min, 37°C). Equal aliquots of RNase-treated and untreated samples were subjected to electrophoresis on 6% acrylamide–8 M urea gels and analyzed by autoradiography.

RESULTS

Lack of effect of Vif on the protein composition of HIV particles. Virus particles produced from nonpermissive cell types such as H9 or PBMC in the absence of Vif are defective and are unable to initiate productive replication even in permissive target cells (65, 66). To assess the impact of Vif on the protein composition of virions produced from restrictive, semi-permissive, and permissive cell types, we performed a comparative analysis of virions derived from transiently transfected HeLa cells (permissive) and from various T-cell lines (Jurkat [permissive], A3.01 [semipermissive], H9 [nonpermissive]) and PBMC (nonpermissive) infected with virus stocks pseudotyped with the VSV glycoprotein G. To prevent subsequent second rounds of infection, we used *env*-defective variants of NL4-3 either expressing functional Vif or carrying an inactive *vif* gene. Furthermore, to avoid possible contamination of the progeny virus population with residual input virus, virus inocula were removed 5 h after infection, and cells were washed once with complete RPMI and cultured in fresh RPMI-FBS for 24 h. Then, culture supernatants were once again discarded and replaced with fresh RPMI-FBS. Thus, only virus produced between days 2 and 3 postinfection was used for this analysis. Virus-containing supernatants were cleared by centrifugation and filtered to remove residual cellular debris. Virus particles were pelleted through a 20% sucrose cushion to remove soluble proteins. Viral pellets were dissolved in sample buffer and separated by SDS-PAGE. Immunoblot analysis was performed using an HIV-positive patient serum (Fig. 1, APS) and normalized for comparable levels of viral integrase using an integrase-specific antibody (Fig. 1, α-Int). As can be seen in Fig. 1, the protein composition of viruses, in particular the relative amounts of Gag processing intermediates, varied in a host cell-dependent manner. However, the presence or absence of Vif did not have a measurable effect on the protein composition of viruses derived from the same host. Thus, Vif does not

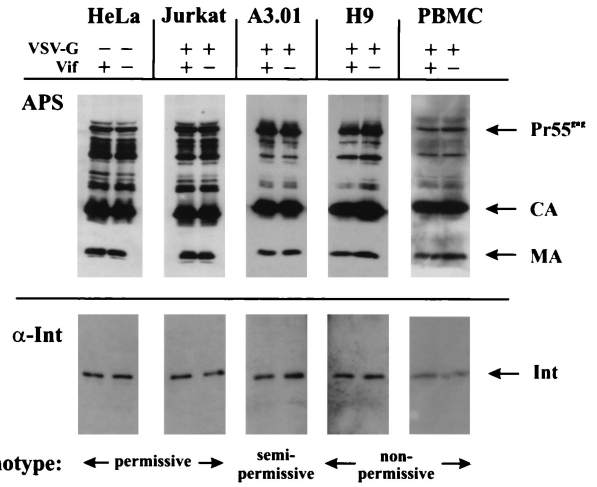


FIG. 1. Vif does not affect the protein composition of HIV virions. Virus preparations from HeLa cells were produced by transient transfection with pNL4-3 or pNL4-3Vif(−). Viruses from T-cell lines and PBMC were obtained from single-cycle-infected cultures. For single-round infection of T-cell lines and PBMC, virus stocks containing the VSV G protein were produced in HeLa cells by cotransfection of an *env*-deficient pNL4-3 variant (pNLenv-1) and pHCMV-G as described in Materials and Methods. Cells were infected with concentrated virus stocks for 5 h before residual input virus was removed. As a further precaution against contamination by input virus, virus produced within the first 24 h after infection was discarded. Only virus produced thereafter was used for immunoblot analysis. Virus was normalized for equal reverse transcriptase activity and analyzed by immunoblotting using an HIV-positive patient serum (APS) or an antiserum to integrase (α-Int) (29). Viral proteins are identified on the right.

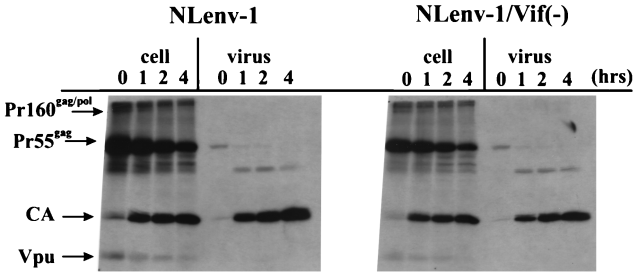


FIG. 2. Vif has no discernible effect on synthesis or maturation of viral proteins or on virus release in HIV-infected H9 cells. H9 cells were single-cycle infected with wild-type (NLenv-1) or Vif-defective [NLenv-1/Vif(−)] variants of the *env*-defective NL4-3 isolate pseudotyped with the VSV-G envelope as described for Fig. 1. Twenty-four hours after infection, cells were metabolically labeled for 1 h with [^{35}S]methionine and chased for up to 4 h. Equal aliquots of cells and supernatants were harvested and subjected to immunoprecipitation with an HIV-positive patient serum. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by fluorography. Proteins are identified on the left.

have an overall impact on the protein composition of virus particles.

Vif has no discernible effect on synthesis, maturation, or release of major capsid proteins from HIV-infected H9 cells. To assess possible effects of Vif on the maturation kinetics of viral proteins in HIV-infected cells, H9 cells were infected with VSV-G-pseudotyped wild-type or Vif-defective virus stocks. As before, *env*-defective variants of NL4-3 [pNLenv-1 and pNLenv1/Vif(−)] were used to avoid second-round infection by wild-type virus. Twenty-four hours after infection, cells were metabolically labeled for 1 h with [^{35}S]methionine and chased for up to 4 h, as indicated in Fig. 2. Equal aliquots of cells and supernatants were harvested and subjected to immunoprecipitation with an HIV-positive patient serum. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by fluorography (Fig. 2). Consistent with the results from Fig. 1, no kinetic differences in the processing of Pr55^{gag} or Pr160$^{gag/pol}$ or in the appearance of the mature p24^{CA} were seen in infected H9 cells in the presence or absence of Vif. While this analysis does not rule out possible subtle differences in the release of NC, P1, or p6 from the Pr55^{gag} precursor, such differences in virus maturation seem unlikely, since one of the slowest Gag processing events, i.e., the release of P2 from the p25 precursor, did not seem to be affected, based on comparison of the p24/25 ratios from wild-type and Vif-defective viruses (data not shown).

Vif is present in virus preparations derived from nonpermissive donor cells. In our previous work, we reported the efficient packaging of Vif in HeLa cell-derived virions (34). However, recent reports have suggested that HIV-1 virions produced in restrictive cell types such as H9 contain only very low levels of Vif (16, 56). Since HeLa cells are permissive and do not depend on Vif for the production of infectious virions, we set out to verify our previous results by analyzing viruses derived from the nonpermissive H9 cell line acutely infected with NL4-3 virus. Virus-containing supernatants were harvested near the peak of infection and concentrated by ultracentrifugation. Concentrated virus was purified on a linear 10 to 50% sucrose gradient as described in Materials and Methods. Individual fractions were separated by SDS-PAGE and analyzed by immunoblotting for the presence of viral proteins using an HIV-positive patient serum (Fig. 3, APS), antibodies to HIV-1 integrase (α-Int) as a marker for viral cores, or a Vif-specific antiserum (α-Vif). Small amounts of soluble CA proteins were identified in fractions 1 and 2, while the main peak containing virus was found in fractions 8 to 11. Similarly, both integrase and Vif proteins peaked in fractions 8 to 10, although smaller amounts of Vif and Int were also detectable in the adjacent gradient fractions. The finding that Vif is indeed associated with HIV particles derived from H9 cells validates our previous observation in HeLa cells and suggests that packaging of Vif is cell type independent.

Mutation of nucleocapsid zinc finger domain abolishes Vif incorporation into virions. We and others have previously shown that Vif can be found in association with cell-free viruses (10, 34, 39). However, by some accounts, the efficiency of Vif packaging is low (16) and dependent on the intracellular expression levels (56). Furthermore, Vif was found to be packaged into murine leukemia virus (MLV) virions (10), suggesting that Vif packaging may be nonspecific, and its functional significance has thus been questioned. Two recent reports suggest that Vif can associate with viral genomic RNA in infected cells (15, 72). In addition, Vif was found to bind to the nucleocapsid domain of Pr55^{gag} precursors (8, 33). To investigate the possible significance of the Vif-NC interaction for the packaging of Vif into viral particles, we made use of the fact that mutations in the nucleocapsid (NC) zinc finger domains significantly impair RNA packaging (4, 14, 17, 27, 28). Due to this impairment of RNA packaging, NC zinc finger mutants are noninfectious and cannot be transmitted to H9 cells by single-cycle infection techniques. We therefore studied the impact of NC zinc finger mutations on Vif packaging in HeLa cells by comparing Vif incorporation into wild-type NL4-3 virions with

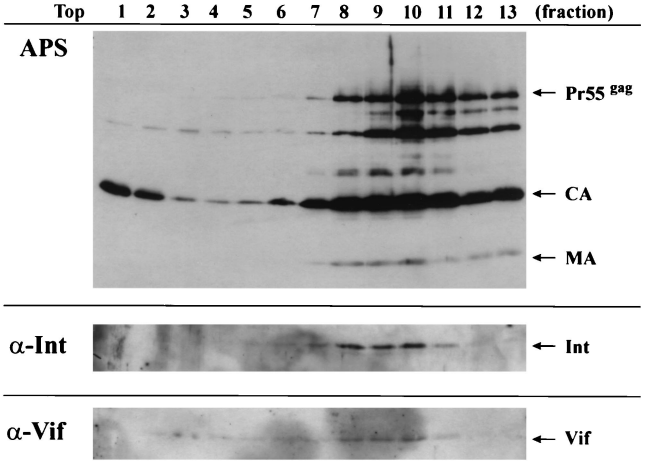


FIG. 3. Vif is present in virus preparations derived from nonpermissive H9 cells. H9 cells were infected with NL4-3. Virus was harvested near peak infection, filtered through 0.45-μm filters to remove cellular debris, and concentrated by ultracentrifugation. Concentrated virus stocks were subjected to linear 10 to 50% sucrose gradient centrifugation as described in Materials and Methods. Individual gradient fractions were analyzed by immunoblotting using an HIV-positive patient serum (APS) or antibodies to integrase (α-Int) or Vif (α-Vif). Viral proteins are identified on the right.

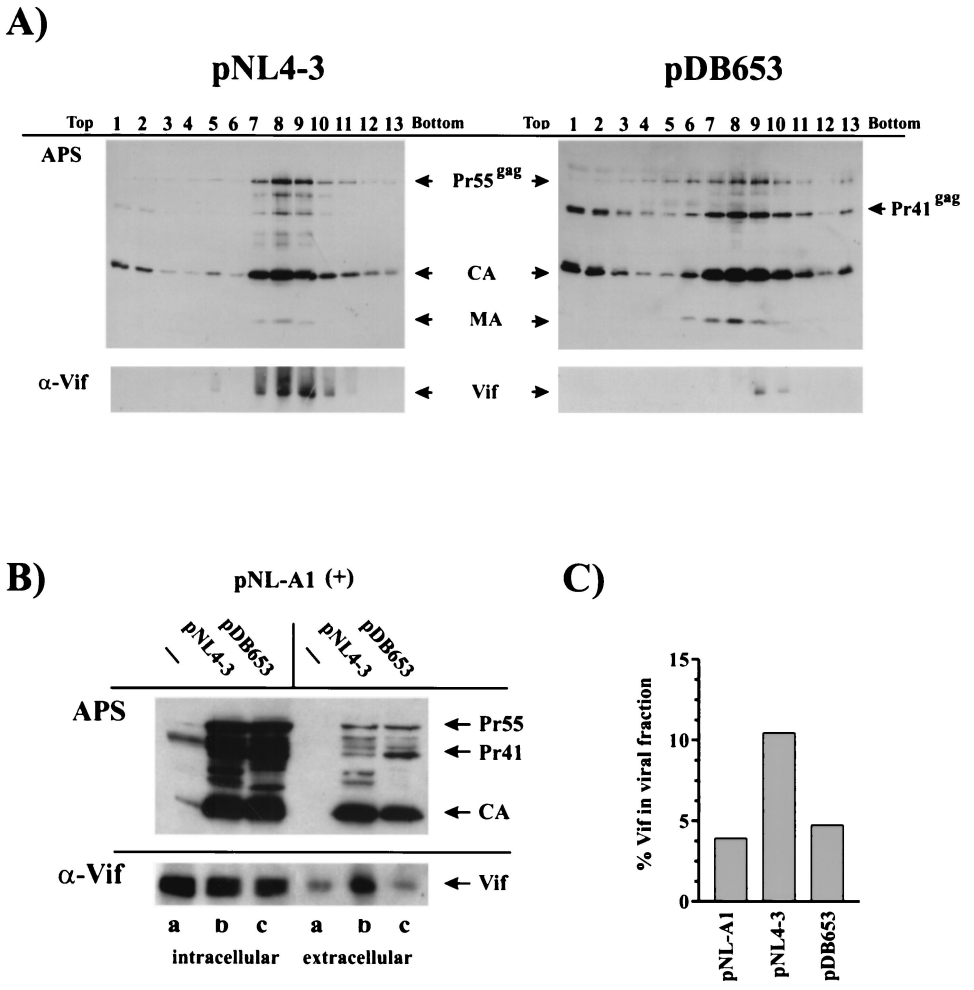


FIG. 4. Mutation of the nucleocapsid zinc finger domain abolishes Vif incorporation into virions. (A) HeLa cells were transiently transfected with plasmid DNAs encoding wild-type HIV-1 (pNL4-3) or a nucleocapsid zinc finger mutant of NL4-3 (pDB653). Virus-containing supernatants were harvested 48 h after transfection, concentrated, and subjected to 10 to 60% linear sucrose gradient centrifugation. Individual gradient fractions were collected and subjected to immunoblotting using an HIV-positive patient serum (APS) or a Vif-specific antiserum (α-Vif). (B) HeLa cells were transfected with the Vif expression vector pNL-A1 (lanes a) or contransfected with pNL-A1 plus pNL4-3 (lanes b) or the zinc finger mutant pDB653 (lanes c). Virus-containing supernatants were harvested 48 h after transfection and pelleted through a cushion of 20% sucrose. Cell lysates and viral pelleted fractions were subjected to immunoblot analysis using an HIV-positive patient serum (APS) or a Vif-specific antiserum (α-Vif). (C) Bands corresponding to Vif in panel B were quantified by densitometric scanning, and the proportion of Vif identified in the pooled gradient fractions was calculated as percentage of total intra- and extracellular Vif.

a zinc finger mutant, pDB653, in which all cysteine residues of the NC zinc finger domains were changed to serine residues. This mutant was significantly impaired in packaging of viral genomic RNA (30). HeLa cells were transfected with pNL4-3 or the zinc finger mutant pDB653. Virus-containing supernatants were harvested 48 h after transfection. Concentrated virus stocks were prepared as described in Materials and Methods and subjected to centrifugation on linear 10 to 60% sucrose gradients. Individual gradient fractions were separated by SDS-PAGE and analyzed by immunoblotting using an HIV-positive patient serum (Fig. 4A, APS) or a Vif-specific antibody (Fig. 4A, α-Vif). Wild-type virus preparations contained significant levels of Vif that comigrated with the peak virus fractions. In contrast, only low levels of Vif were found in virus preparations from cells expressing the NC zinc finger mutant pDB653 (Fig. 4A, right panel). These results indicate that mutation of the NC zinc finger domain potently interferes with the packaging of Vif.

To quantify the impact of the NC zinc finger mutations on Vif packaging, we calculated the ratio of incorporation efficiency by comparing the relative amounts of intracellular versus intravirion Vif. To rule out possible differences in the Vif expression levels between NL4-3 and pDB653 variants, in this experiment Vif was expressed in *trans* from the Vif expression plasmid pNL-A1, which does not produce virus particles (62). Thus, HeLa cells were cotransfected with pNL-A1 along with pNL4-3 (Fig. 4B, lanes b) or pDB653 plasmid DNAs (Fig. 4B, lanes c). As a control, HeLa cells were transfected with pNL-A1 plasmid DNA alone (Fig. 4B, lanes a). Cell-free supernatants were harvested 48 h after transfection and subjected to centrifugation through a 20% sucrose cushion. Pelleted material was solubilized in sample buffer. Aliquots of the

cell lysates (5% of total) and viral pellets (20% of total) were separated by SDS-PAGE and analyzed by immunoblotting using an HIV-positive patient serum (Fig. 4B, APS) or a Vif-specific antiserum (Fig. 4B, α-Vif). Bands corresponding to intra- and extracellular Vif were quantified by densitometric scanning, and the amount of Vif in the pooled gradient fractions was calculated as a percentage of total intra- and extracellular Vif (Fig. 4C). As can be seen in Fig. 4C, approximately 4% of total Vif was found in pooled gradient fractions from pNL-A1-transfected cells. This presumably reflects the level of nonspecific association of Vif with secreted membrane vesicles. In contrast, more than 10% of Vif was found in NL4-3 virus preparations. Importantly, the amount of Vif identified in NC zinc finger mutant virus preparations was reduced to near background levels (Fig. 4C, compare pNL-A1 and pDB653) despite similar levels of intracellular Vif (Fig. 4B, compare lanes b and c) and comparable levels of extracellular virus. Similar results were observed with a separate nucleocapsid zinc finger mutant, pRB73-B-H23C/H44C (not shown) (27). Thus, mutation of the NC zinc finger domains blocks the association of Vif with HIV particles independently of the intracellular expression levels.

Packaging of Vif into viral particles requires packaging of viral genomic RNA. The inability of Vif to associate with the nucleocapsid mutant viruses could be a consequence of the reduced ability of these viruses to package viral genomic RNA or result from the inability of Vif to interact with the mutant nucleocapsid protein itself. To address this question, we analyzed the efficiency of Vif incorporation into an HIV-1 RNA-packaging mutant, C-Help (42). Unlike the NC zinc finger mutants, C-Help does not carry mutations in the viral *gag* gene but lacks a putative RNA-packaging motif upstream of the Gag coding region and, in addition, lacks both viral LTRs (Fig. 5A). Thus, the lack of packaging of viral genomic RNA is due to a defect in the viral RNA rather than the viral capsid. Analysis of C-Help virus preparations by endogenous RT assay did not reveal detectable levels of viral RNAs (see Fig. 7). HeLa cells were transiently transfected with C-Help plasmid DNA as described above. Virus-containing supernatants were harvested 48 h after transfection and concentrated by ultracentrifugation. Concentrated virus preparations were either analyzed directly (Fig. 5, lane b) or subjected to sucrose step gradient centrifugation (lanes c to e). Three equal fractions were collected from the step gradient, as indicated in the diagram in Fig. 5. Whole-cell lysates (lane a) and viral fractions were subjected to immunoblot analysis using an HIV-positive patient serum or a Vif-specific antiserum. Only small amounts of Vif were detectable in concentrated virus preparations (lane b), which were below the level of detection following step gradient centrifugation. Quantitation of the Vif-specific bands from cell lysates and concentrated virus preparations (lanes a and b) was done, as shown in Fig. 4C. The amount of Vif identified in the concentrated virus preparation (lane b) corresponded to approximately 3.5% of total Vif, which is comparable to the level of nonspecific Vif secretion observed in HeLa cells in the absence of virus production or in cells producing the NC zinc finger mutant (Fig. 4C, pNL-A1 and pDB653). The level of Vif found in C-Help virus preparations is well below the levels found in wild-type NL4-3 preparations, which were consistently in excess of 10% of total Vif. Thus, deletion of the viral LTRs and a putative RNA-packaging signal of the viral genomic RNA severely restricted Vif incorporation into virions. These results suggest that packaging of Vif into virus particles occurs concomitant with the packaging of viral genomic RNA. These results also suggest that the impact of NC zinc finger mutations of Vif packaging is not the result of a loss of interaction between Vif and NC but a consequence of the reduced RNA packaging exhibited by those mutants.

Vif is a component of viral nucleoprotein complexes. To determine the location of Vif within virions, we performed a series of experiments that compared the sensitivity of Vif and other known virion components to detergent treatment. Detergent extraction of virus preparations maintains the viral core structure (38) but eliminates the viral envelope and its associated proteins, including gp41 and MA, and is expected to disrupt cellular membrane vesicles. Proteins that were packaged through passive diffusion and are located in the lumen of the virions should be separated from the virus cores by detergent treatment as well. To measure potential differences between viruses derived from permissive and restrictive hosts, we analyzed virus preparations derived from either pNL4-3-transfected HeLa cells or acutely infected H9 cells.

Concentrated virus preparations from HeLa and H9 cells were subjected to step gradient centrifugation in the absence (Fig. 6, untreated) or presence of Triton X-100 (Fig. 6, X100) as detailed in Materials and Methods. Individual fractions from each step gradient were subjected to immunoblot analysis using an HIV-positive patient serum (Fig. 6, top panels) or antibodies to gp41, integrase, nucleocapsid, or Vif. As expected, untreated viruses accumulated at the 20 to 60% sucrose interphase (lanes 3, untreated), with only minor amounts of soluble viral proteins detectable in the soluble fraction (lanes 1, untreated) and the 20% sucrose fraction (lanes 2, untreated). Upon detergent treatment, proteins associated with the viral envelope, i.e., gp41 and MA, were quantitatively extracted from the virions, as shown by their displacement from fraction 3 to fraction 1 (lanes 1, X100). The viral capsid was also sensitive to detergent treatment, and a major portion of CA protein was found in the soluble fraction in both HeLa and H9 cell-derived virus preparations. NC, a component of the viral nucleoprotein complex, exhibited partial sensitivity to detergent extraction, with approximately 40% of the virus-associated protein remaining associated with the viral cores. Integrase, another component of nucleoprotein complexes, and residual unprocessed $Pr55^{gag}$ protein were insensitive to detergent treatment and remained mainly associated with the viral cores. Interestingly, Vif was also largely resistant to detergent extraction and consistently copurified with viral integrase and $Pr55^{gag}$ molecules. Of note, there was no obvious difference in the detergent sensitivity of viruses derived from permissive HeLa and restrictive H9 cells. These data rule out the possibility that Vif is nonspecifically attached to the viral envelope or is located in the lumen of virus particles. Furthermore, these results are inconsistent with an association of Vif with cellular membrane vesicles, which have been reported to be a major source of contamination of gradient-purified virus preparations (5, 16, 23). Our results therefore provide strong evidence that Vif is a virion component and suggest that Vif is a component of the viral core structure.

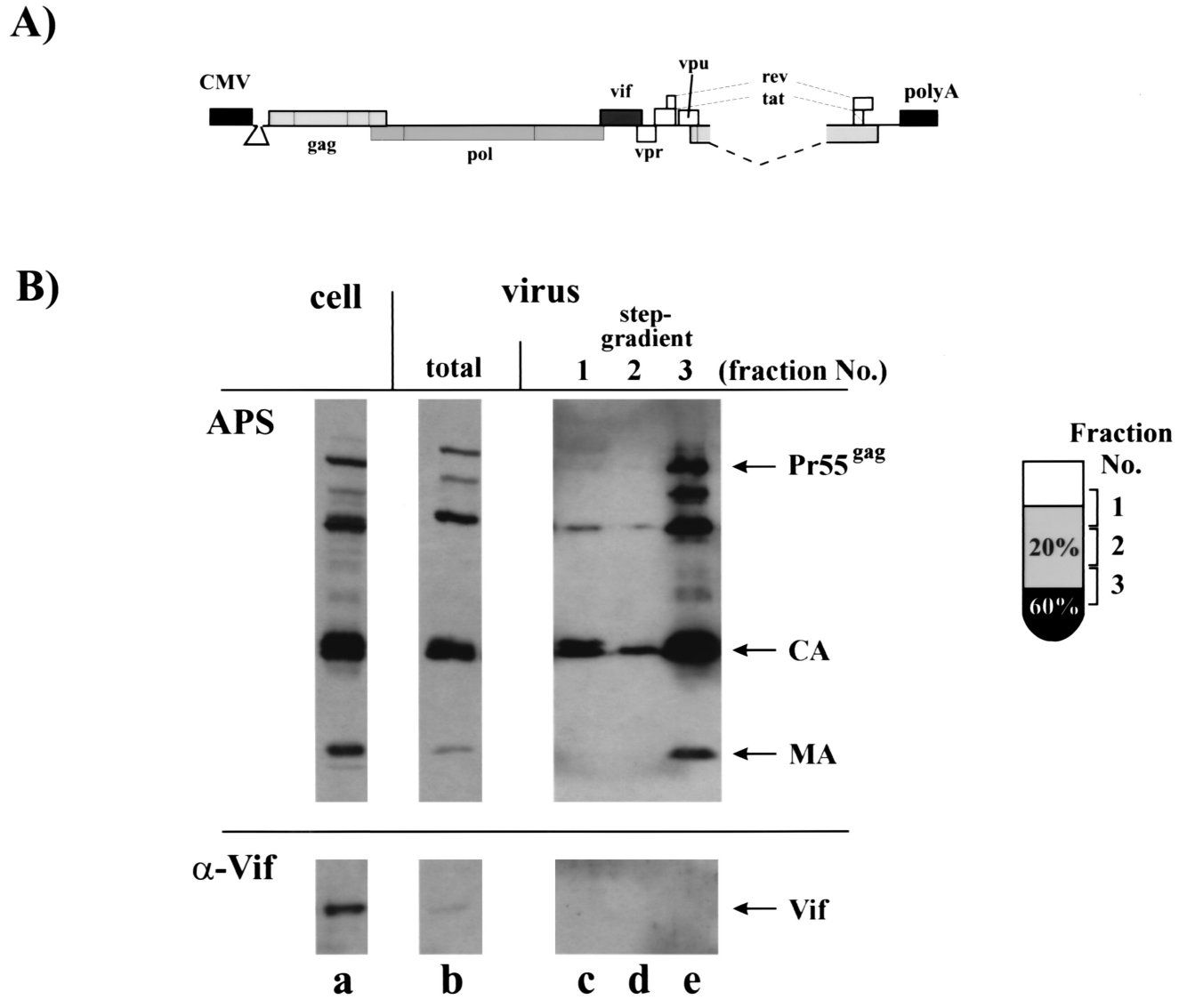


FIG. 5. Efficient packaging of Vif requires viral RNA. (A) Schematic outline of the structure of the RNA-packaging mutant pC-Help (42). The plasmid lacks both viral LTRs and carries a deletion upstream of *gag* (indicated by the triangle) which eliminates a putative RNA-packaging signal. In addition, the plasmid carries a deletion in the *env* gene (indicated by a broken line). polyA, poly(A) addition site. (B) HeLa cells were transfected with pC-Help plasmid DNA. Virus-containing supernatants were harvested 48 h after transfection and concentrated by ultracentrifugation. Concentrated virus was either analyzed directly (lane b) or subjected to sucrose step gradient centrifugation as described in Materials and Methods (lanes c to e). Three equal fractions were collected as indicated in the diagram on the right. Cell lysates (lane a) and viral fractions were separated by SDS–12.5% PAGE and subjected to immunoblot analysis using an HIV-positive patient serum (APS) or a Vif-specific antiserum (α-Vif). Vif-specific bands in lanes a and b were quantified by densitometric scanning as in Fig. 4. Viral proteins are identified on the right.

Active viral reverse transcription complexes are resistant to detergent treatment. To assess the sensitivity of virion-associated reverse transcriptase to detergent treatment, we performed a standard RT assay on individual step gradient fractions of the H9 cell-derived virus shown in Fig. 6 (Fig. 7A). In addition, step gradient fractions of HeLa cell-derived C-Help virus described in Fig. 5 as well as the NC mutant DB653 employed in Fig. 4 were analyzed in parallel. In untreated virus preparations, reverse transcriptase activity in wild-type NL4-3 was almost exclusively limited to fraction 3, attesting to the efficiency of the step gradient procedure in separating viral from soluble proteins. In contrast, step gradient fractionation of detergent-treated NL4-3 resulted in the release of almost half of the virus-associated reverse transcriptase into the soluble fraction. Only about 35% of the virus-associated enzyme activity remained in fraction 3. In C-Help and DB653 virus preparations, we observed some soluble reverse transcriptase activity even in the absence of detergent treatment. It is unclear whether this reflects an increased instability of these viruses or is due to the secretion of soluble reverse transcriptase from the transfected HeLa cells. Nevertheless, more than 70% of the reverse transcriptase activity was associated with the virus-containing fraction 3 in these virus preparations.

To ascertain that detergent treatment of viruses did not

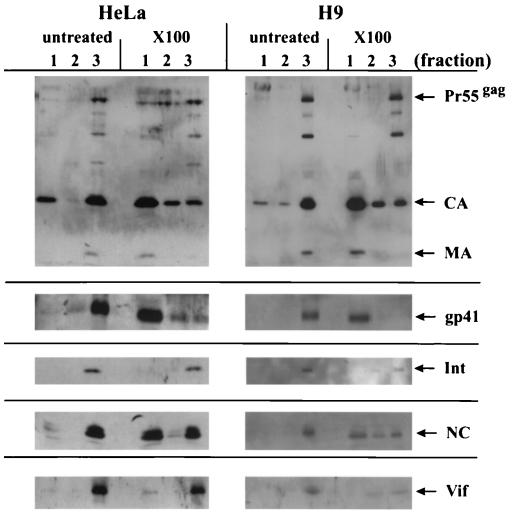


FIG. 6. Vif is resistant to detergent extraction of virus derived from HeLa and H9 cells. HeLa-derived virus stocks were prepared by transient transfection of HeLa cells with pNL4-3 DNA as described for Fig. 4. For the preparation of virus stocks from H9 cells, H9 cells were infected with the NL4-3 isolate. Virus-containing supernatants were harvested near the peak of the infection. HeLa- and H9-derived viruses were concentrated by ultracentrifugation and subjected to step gradient purification as described for Fig. 5. To assess the detergent sensitivity of viral components, 50% of the virus preparation was subjected to step gradient centrifugation in the presence of Triton X-100 (X100). The remaining virus was left untreated (untreated). Three fractions containing soluble proteins (fraction 1), a buffer fraction (fraction 2), and the virus-containing fraction (fraction 3) were harvested as in Fig. 5. Individual gradient fractions were subjected to immunoblot analysis using an HIV-positive patient serum (top panels) or antibodies to gp41, integrase (Int), nucleocapsid (NC), or Vif, as indicated on the right.

destroy viral nucleoprotein complexes, we performed endogenous RT assays to measure the synthesis of minus-strand strong-stop cDNA [(−)ssDNA]. This assay requires a functional nucleoprotein complex including viral genomic RNA, a $tRNA^{Lys}$ primer, and reverse transcriptase. The primary product of the endogenous RT assay reaction is a 253-nucleotide RNA-DNA hybrid from which the RNA component can be removed by treatment with RNaseA, resulting in a 181-base cDNA (Fig. 7B). Details of the assay are described in the Materials and Methods section. The synthesis of (−)ssDNA was measured for each of the fractions of the step gradients shown in Fig. 7A (Fig. 7B). To discriminate between cDNA synthesis from unspliced viral genomic RNA and nonspecifically packaged spliced viral mRNA, we included a synthetic oligonucleotide (Z85) with the ability to prime cDNA synthesis from a position downstream of the major 5′ splice site (positions 337 to 354 on the viral RNA). The product of the Z85-primed reverse transcription is expected to be 354 nucleotides long and specific for unspliced genomic RNA. As expected, (−)ssDNA from untreated virus preparations was restricted to the viral fraction (fraction 3) of the step gradient (Fig. 7A, untreated). Similarly, Z85-primed cDNA was detectable exclusively in fraction 3 in untreated virus preparations. Interestingly, in the detergent-treated NL4-3 preparation, (−)ssDNA and Z85-primed cDNA were similarly restricted to fraction 3. Nonspecific cDNA synthesis was apparent as a background smear in fraction 1, indicating the presence of nonspecific RNA in the virus preparations. However, despite the presence of significant amounts of reverse transcriptase activity in fractions 1 and 2 (Fig. 7A, X100), no (−)ssDNA-specific products were observed in these fractions in the detergent-treated virus preparation. The amounts of (−)ssDNA synthesized relative to the Z85-primed products were identical for untreated and detergent-treated viruses, suggesting that synthesis of (−)ssDNA occurred to a large extent, if not exclusively, from unspliced viral genomic RNA. Furthermore, the absence of any Z85-primed products in fractions 1 and 2 of untreated and detergent-treated virus preparations attests to the absence of viral genomic RNA from these fractions. These results confirm that viral nucleoprotein complexes capable of directing the synthesis of (−)ssDNA are resistant to detergent extraction. The resistance of both Vif and active reverse transcription complexes to detergent extraction is additional evidence that Vif is, in fact, an integral component of the viral nucleoprotein complex.

Analysis of the RNA-packaging-defective C-Help and DB653 viruses by endogenous RT assay did not reveal any (−)ssDNA products despite the presence (see Fig. 7A) of significant levels of virion-associated RT activity. This is expected for the C-Help virus due to the lack of sequences required for (−)ssDNA synthesis, including the 5′ LTR and the $tRNA^{Lys}$ primer binding site (see Fig. 7C). The DB653 NC mutant, on the other hand, contains all the RNA sequences necessary for (−)ssDNA synthesis. Therefore, the absence of (−)ssDNA in DB653 suggests the absence of viral genomic RNA in these virus preparations. Importantly, no Z85-derived cDNA products were observed for either the C-Help or DB653 virus even though both virus genomes contain the sequences required for Z85-primed cDNA synthesis. These results are best explained by the lack of viral genomic RNA in both virus preparations.

Deletion of a central basic domain in Vif abolishes packaging into progeny virions. The above data strongly suggest that Vif is packaged into virions through a specific interaction with viral genomic RNA. To identify domains in the Vif protein required for virion incorporation, we constructed a series of in-frame deletions in the *vif* gene (Fig. 8A). All Vif variants were constructed by PCR-directed mutagenesis as described in Materials and Methods and cloned into the backbone of either pNL-A1 or pNL4-3. Metabolic labeling of transiently transfected HeLa cells followed by immunoprecipitation demonstrated that all Vif variants, with the exception of VifΔB, were stably expressed and efficiently recognized by the Vif-specific antibody (Fig. 8B). The low abundance of a VifΔB-specific protein band (Fig. 8B, lane 3) was not due to metabolic instability of this particular Vif variant but was a consequence of the deletion of the major epitope recognized by our Vif-specific antibody. This is evidenced by the fact that addition of a Flag epitope tag resulted in the efficient recognition of $VifΔB_{Flag}$ by the M2 monoclonal antibody (Fig. 8B, lane 8). All mutants were found to replicate in semipermissive A3.01 cells with kinetics similar to that of a Vif-defective variant and did not support productive infection of nonpermissive H9 cells (not shown). The fact that deletions in all regions of Vif were associated with loss of function suggests that Vif has multiple

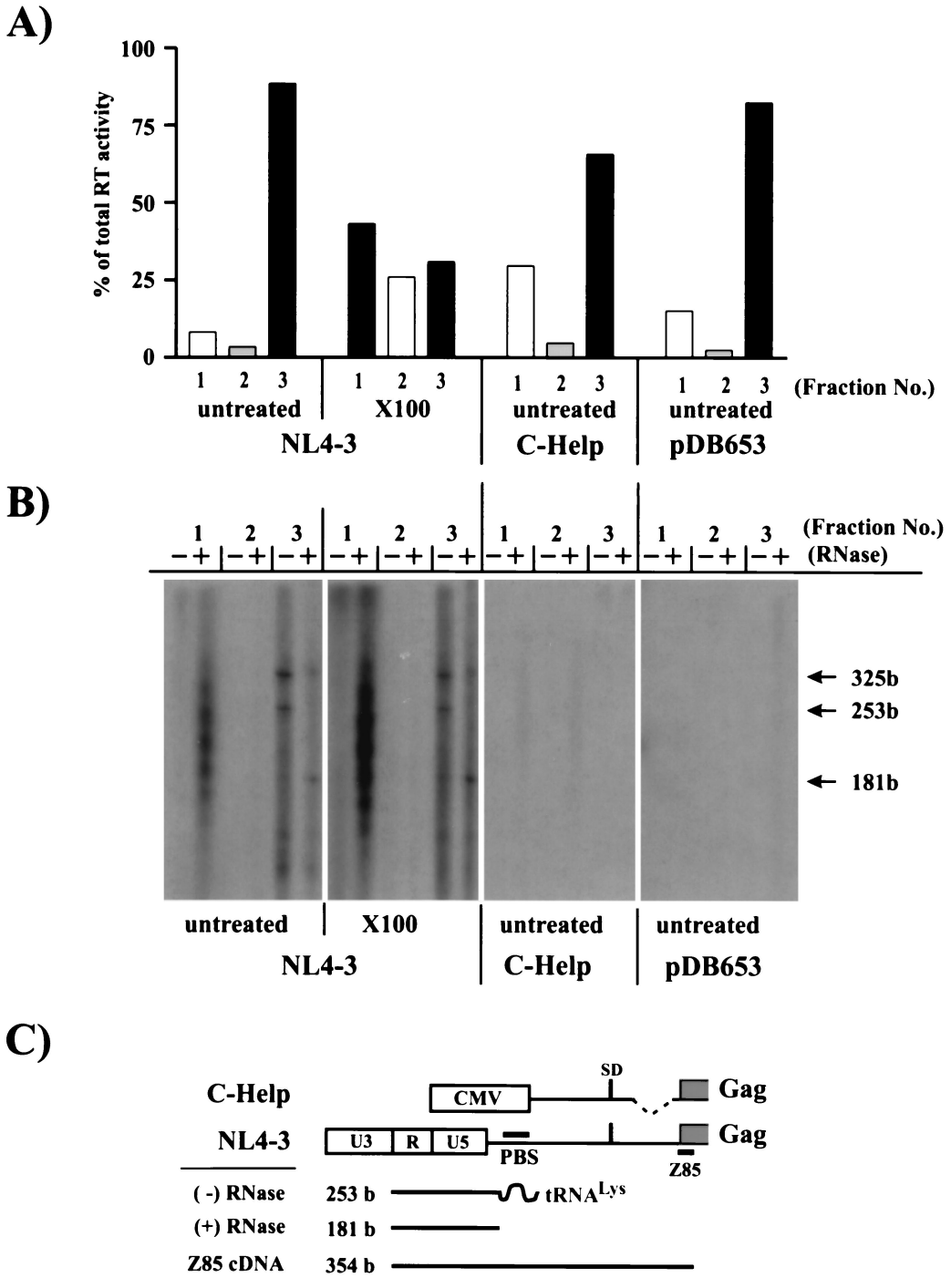
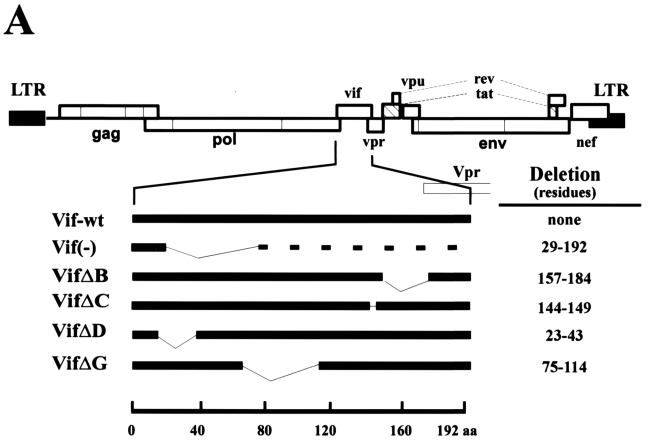


FIG. 7. Viral genomic RNA and the reverse transcription complex are insensitive to detergent treatment. (A) Step gradient fractions of virus preparations derived from H9 cells (Fig. 6) or from HeLa cells transfected with pC-Help (Fig. 5B) or pDB653 were examined for reverse transcriptase activity using a conventional RT assay (67). Values are expressed as a percentage of the total reverse transcriptase activity. (B) All fractions analyzed in panel A were subsequently analyzed by an endogenous reverse transcriptase assay as described in Materials and Methods. The predicted size of the $tRNA^{Lys}$-derived (−)ssDNA is 253 nucleotides (b). Removal of the tRNA component from the (−)ssDNA by RNase treatment is expected to reduce its size to 181 nucleotides. A synthetic oligonucleotide, Z85, was included in the reactions to control for the presence of unspliced viral genomic RNA in individual fractions. The predicted size of the Z85-primed cDNA product is 354 nucleotides. (C) Schematic outline of the predicted products from the endogenous RT assay. PBS, binding site for the $tRNA^{Lys}$ primer; SD, splice donor site. A deletion in C-Help eliminating a putative RNA-packaging signal is indicated by a broken line.



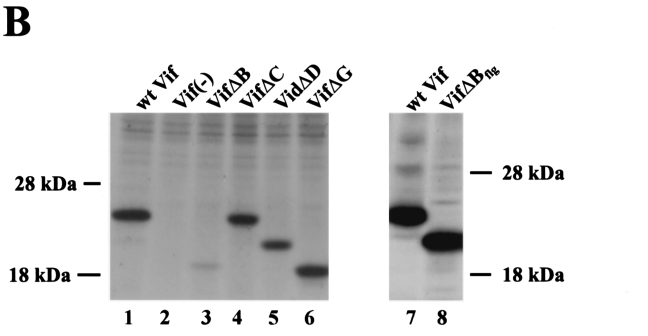


FIG. 8. Deletions in Vif do not affect Vif expression levels. (A) Schematic representation of deletions introduced into the *vif* gene. Construction of the individual mutants is described in Materials and Methods. Deleted regions (amino acid positions) in Vif are denoted on the right. (B) HeLa cells were transfected with pNL-A1 (wt Vif), pNL-A1Vif(−), or individual deletion mutants as indicated. Twenty-four hours after transfection, cells were metabolically labeled for 90 min with [^{35}S]methionine. Cell lysates were subjected to immunoprecipitation with a Vif-specific antibody followed by SDS–12.5% PAGE (lanes 1 to 6). In a similar experiment, cells were transfected with either pNL-A1 (wt Vif) or a Flag epitope-tagged variant of VifΔB, pNL-A1/VifΔB$_{Flag}$. Cells were labeled as before and precipitated with either a Vif-specific antibody (wt Vif, lane 7) or the epitope tag-specific M2 monoclonal antibody (VifΔB$_{Flag}$, lane 8). Proteins were subjected to SDS–12.5% PAGE and visualized by fluorography.

functional domains, all of which are important for regulation of viral infectivity.

Packaging of Vif variants into HIV particles was determined in HeLa cells. Since the *vif* gene overlaps the Int and Vpr open reading frames at the N and C termini, respectively, deletions in those regions of Vif are likely to affect the function of the overlapping gene products. To avoid potential interference by mutations in Int or Vpr with our packaging assay, Vif variants were expressed in *trans* from pNL-A1-based vectors together with the Vif-defective pNL4-3 (pNL4-3ΔVif). Purified virus preparations were obtained by step gradient centrifugation. For quantitative extraction of Vif from cells, whole-cell lysates were prepared by first lysing cells in CHAPS-DOC lysis buffer, followed by boiling of residual insoluble material in sample buffer. Pooled cell fractions were compared to the viral extracts by immunoblotting using a Vif-specific antiserum (Fig. 9A, α-Vif) or an HIV-positive patient serum (Fig. 9A, APS). Vif-specific protein bands were quantified by densitometric scanning (Fig. 9B). Consistent with the results in Fig. 4C, approximately 12% of the total wild-type Vif protein was associated with virus particles. Similar results were observed for Vif mutants lacking the C-terminal basic domain (VifΔB) or a highly conserved motif ($S_{144}LQYLA_{149}$) in Vif (VifΔC), suggesting that the C-terminal domain in Vif does not contain signals important for Vif packaging (not shown). Interestingly, deletion of an N-terminal motif in Vif (residues 23 to 43, VifΔD) doubled its packaging efficiency to nearly 23% of total Vif. In striking contrast, deletion of residues 75 to 114 (VifΔG) virtually abolished Vif packaging. Thus, Vif contains sequences that are important for packaging into virions, providing additional evidence that packaging of Vif is a specific process.

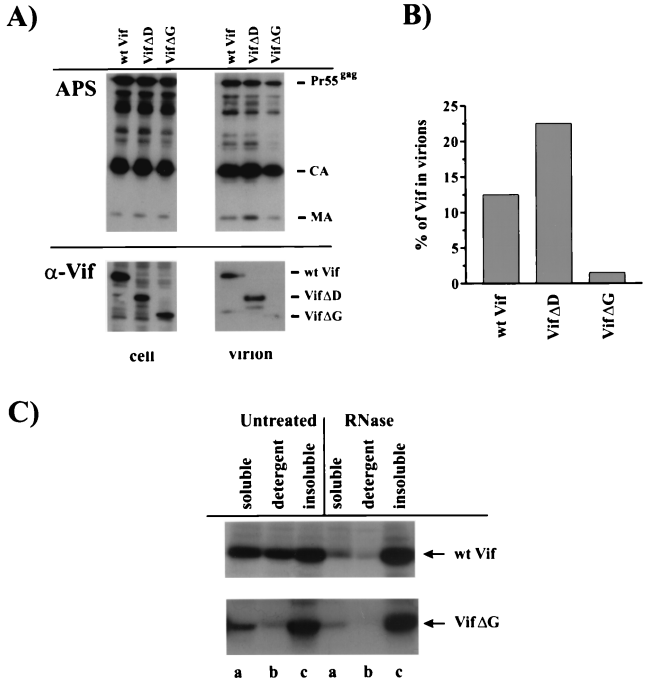


FIG. 9. Deletion of a central domain in Vif abolishes packaging into virions. (A) To assess the impact of deletions in Vif on packaging into virions, HeLa cells were cotransfected with pNL4-3Vif(−) and either pNL-A1 (wt Vif), pNL-A1/VifΔD (VifΔD), or pNL-A1/VifΔG (VifΔG). Cells and virus-containing supernatants were harvested 48 h posttransfection. Virions were purified and concentrated by sucrose step gradient centrifugation as described in Materials and Methods. Defined fractions of cell lysates and viral pellets were subjected to SDS–12.5% PAGE followed by immunoblotting with an HIV-positive patient serum (APS) or a Vif-specific antibody (α-Vif). (B) Intracellular and virus-associated Vif proteins detected in panel A were quantified using the FujiX Image Gauge software. The amount of Vif associated with virions was calculated as a percentage of total intra- and extracellular Vif. (C) HeLa cells were transfected with pNL-A1 (wt Vif) or pNL-A1/VifΔG (VifΔG). In addition to the expression of Vif, both plasmids encode authentic viral mRNAs for the expression of Vpr, Tat, Rev, Vpu, Env, and Nef (62). Cells were metabolically labeled for 90 min as described for Fig. 8B. Cells were fractionated into soluble (lanes a), detergent-soluble (lanes b), and detergent-resistant (lanes c) fractions in either the presence or absence of RNase A as described in Materials and Methods. Vif proteins present in individual fractions were precipitated using the Vif-specific polyclonal antibody, separated by SDS–12.5% PAGE, and visualized by fluorography.

The sequence deleted in VifΔG includes one of two highly conserved cysteine residues (C_{114}) as well as a conserved threonine residue (T_{96}) which was reported to be phosphorylated by mitogen-activated protein kinase (68). Both residues are critical for Vif function (40, 68). In addition, VifΔG lacks a conserved block of basic amino acids ($R_{90}KKR_{93}$). To investigate the possibility that RNA association of Vif plays a role in Vif packaging, we compared the subcellular distribution of wild-type Vif and VifΔG by cell fractionation in the presence and absence of RNase A (Fig. 9C). HeLa cells were transfected with pNLA-1 plasmid DNAs encoding wild-type Vif or Vif deletion mutants. Approximately 24 h posttransfection, cells were metabolically labeled for 90 min with [^{35}S]methionine as described elsewhere (34). Cells were then washed once with PBS to remove excess isotope, pelleted, and suspended in PBS or in PBS containing RNase A (0.5 mg/ml). Cells were disrupted by three cycles of freezing and thawing (fraction a), followed by extraction with CHAPS-DOC buffer (fraction b) as reported previously (34). Detergent-resistant material was solubilized by boiling in sample buffer (fraction c). Individual fractions were immunoprecipitated with a Vif-specific antiserum and separated on SDS–12.5% PAGE followed by fluorography (Fig. 9C). Consistent with our previous report (34), fractionation of cells in the absence of RNase resulted in the partitioning of about 45% of wild-type Vif with the detergent-resistant fraction (wt-Vif, lane c, untreated), 35% of Vif was recovered from the soluble fraction (wt-Vif, lane a, untreated), and about 20% of Vif partitioned with the membrane fraction (wt-Vif, lane b, untreated). In contrast, almost 70% of VifΔG was found in the detergent-resistant fraction in the absence of RNase treatment (Fig. 9C, VifΔG, untreated, lane c), with little more than 20% of VifΔG remaining in the soluble fraction (VifΔG, untreated, lane a) and less than 10% in the detergent-soluble fraction (VifΔG, untreated, lane b). Thus, deletion of amino acids 75 to 114 in VifΔG caused a significant change in the biophysical properties of Vif, resulting in significantly reduced solubility of the protein. In the presence of RNase, the relative proportion of soluble and detergent-soluble forms of VifΔG were further reduced, and more than 90% of Vif was now recovered from the detergent-resistant cellular fraction (Fig. 9C, VifΔG, RNase). Surprisingly, RNase treatment also dramatically altered the subcellular distribution of wild-type Vif, which was now indistinguishable from that of VifΔG, since greater than 90% of the protein was recovered from the detergent-resistant cellular fraction (Fig. 9C, wt Vif, RNase). These results suggest that the soluble and membrane fractions of Vif are associated with viral or cellular RNA and that degradation of the RNA by RNase treatment results in subcellular redistribution and association of Vif with highly insoluble cellular organelles, presumably the cytoskeleton (34). The increased resistance of VifΔG to extraction and the similarity between its subcellular distribution and that of wild-type Vif in RNase-treated extracts could be an indication for a reduced affinity of VifΔG for viral or cellular RNA. Consequently, the low abundance of VifΔG in the cytoplasmic and membrane compartments, which include the site(s) of virus assembly, is a likely explanation for the observed lack of packaging of this mutant. These data thus suggest that RNA association of Vif is required at two different stages of the viral incorporation process: (i) it is required to maintain the protein in the proper subcellular compartment, and (ii) it provides a vehicle for proper insertion and localization in the budding virus particle.

DISCUSSION

Despite significant progress in the characterization of Vif-induced defects of HIV virions, the molecular mechanism of Vif-regulated viral infectivity remains unclear. One of the critical yet unresolved issues is from what cellular—or viral—compartment Vif exerts its activity. Even though previous studies have clearly identified Vif within HIV virions (10, 21, 34, 39, 49), its functional relevance has been questioned. Unlike Vpr, which is packaged into HIV particles through an interaction with the p6 component of Gag (12, 36, 37, 47, 71), packaging of Vif was thought to be nonspecific (10, 56). In addition, the relatively low abundance of Vif in virions, which in some reports approached the limit of detection (70), and the notion that levels of Vif packaging can vary depending on the intracellular expression level without affecting viral infectivity have led to the suggestion that virion incorporation of Vif may not be necessary for Vif function (56).

Our data clearly demonstrate the presence of significant amounts of Vif in viruses irrespective of whether the viruses were derived from permissive HeLa cells or restrictive H9 cells. Packaging of Vif is, in fact, specific and is sensitive to mutations in Vif and dependent on the viral nucleocapsid protein as well as viral genomic RNA. Several lines of evidence support this conclusion. First, mutations in the nucleocapsid zinc finger domain reduce Vif packaging to background levels (Fig. 4). Second, removal of an RNA packaging signal on the viral genomic RNA abolished packaging of Vif (Fig. 5). Third, detergent extraction of HIV virions demonstrates that, in contrast to the viral envelope (gp41), matrix (MA), and capsid (CA) components, which are highly sensitive to detergent extraction, Vif and integrase were insensitive to detergent treatment (Fig. 6). Interestingly, while both reverse transcriptase and nucleocapsid proteins were found to be partially sensitive to detergent extraction (Fig. 6 and data not shown), the active reverse transcription complex capable of directing the synthesis of (−)ssDNA was completely resistant to detergent extraction. Despite the fact that more than 70% of NC and reverse transcriptase were removed by detergent treatment, synthesis of (−)ssDNA in our in vitro assay was equally efficient in untreated and detergent-treated virus preparations, indicating that the integrity of viral reverse transcription complexes were not affected by detergent extraction of viral components. This suggests that the reverse transcriptase and NC molecules released by detergent treatment either constitute excessive amounts of these proteins in virions not tightly associated with reverse transcription complexes or reflect the release of these proteins from defective viral particles.

Viral genomic RNA was also found to be resistant to detergent extraction. This is evidenced by the absence in the soluble fractions of detergent-treated virions of a 354-base reverse transcriptase product directed by the internal Z85 primer (Fig. 7A, compare lanes 1 and 2 with lane 3). While our data are consistent with a previous report demonstrating the impact of deletions in the NC zinc finger domain on packaging of Vif into virions in a recombinant baculovirus system (33), our

observation that Vif is more resistant to detergent extraction than NC or reverse transcriptase as well as the absence of Vif in C-Help virus preparations, is more consistent with an association of Vif with viral genomic RNA rather than (or in addition to) NC. Our data therefore clearly show that Vif is not a soluble component of virions, is not attached to the outside of virions, and is not attached to the viral envelope. Packaging of Vif through a nonspecific mechanism such as passive diffusion is thus unlikely.

In agreement with a previous study (56), we found that the absolute amounts of Vif packaged into virions were affected by the intracellular expression levels (not shown). However, the relative amounts of virion-associated Vif appeared to be rather constant and amounted to about 12.5% of total Vif. This expression level-dependent export of Vif might explain the differences reported in the literature for the number of Vif molecules packaged per virion (10, 15, 21, 39). While point mutations in various regions of Vif were not found to affect its packaging (10), we observed that larger deletions near the N terminus and in the center of the protein had a severe impact on Vif packaging. Deletion of an N-terminal segment in VifΔD (residues 23 to 43) doubled its packaging efficiency to 23% (Fig. 9). This deletion was found to increase the intracellular solubility of Vif, presumably by reducing its reported interaction with vimentin (34; unpublished observations) and could explain the increased packaging efficiency of VifΔD. In contrast, deletion of residues 75 to 114 in VifΔG almost completely blocked packaging of the mutant protein (Fig. 9). These results are consistent with a previous report that identified a requirement for residues 68 to 81 in Vif for packaging into virus-like particles in a baculovirus system (33). Interestingly, deletion of the same region in Vif was also shown to eliminate its RNA-binding activity, which is supported by our observation that the subcellular distribution of wild-type Vif but not VifΔG is RNase sensitive (Fig. 9C). These data further support the notion that packaging of Vif into virions involves an interaction with viral genomic RNA.

While our attempts to identify differences in the protein composition of wild-type and Vif-defective viruses revealed subtle, producer cell-dependent variations (Fig. 1), we failed to observe Vif-dependent variations in the viral protein composition. These results are consistent with observations by other groups (15, 21, 45). Also, our pulse-chase analysis in infected H9 cells did not reveal tangible differences in protein synthesis, processing, or release of viral proteins that could explain the reported effects of Vif on the structure or stability of viral cores (32, 46, 55). In addition, previous studies did not find any effects of Vif on the levels of genomic RNA and $tRNA^{Lys}$ (15) or on genomic RNA dimerization or stability of the RNA dimer linkage (25). Thus, while it is conceivable that viral infectivity requires subtle posttranslational modifications of viral components by Vif, which could be catalyzed by intracellular Vif either before or during virus assembly, there is currently no experimental evidence to support such a mechanism. The obvious correlation between the packaging of viral genomic RNA and Vif and the specific association of Vif with viral cores make it tempting to speculate that packaging of Vif is functionally significant and required for infectivity of virions produced in restrictive producer cells. Our observation that approximately 12% of intracellular Vif molecules are packaged into progeny virions suggest that the packaging of Vif occurs with an efficiency very similar to that reported for HIV-1 Env, where only 5 to 15% of the Env precursor gp160 molecules were found to be transported to the cell surface for virion incorporation (67).

Several possible mechanisms can be envisioned to explain how virus-associated Vif could regulate viral infectivity. First, it is conceivable that due to its affinity to viral RNA and Gag, Vif has a critical role in stabilizing viral nucleoprotein complexes. The function of Vif would therefore be to facilitate proper assembly and/or maturation of components of the viral cores. Accordingly, the absence of Vif would result in unstable, defective cores with reduced ability for efficient cDNA synthesis. Such a mechanism would be consistent with the observation that Vif-defective particles exhibit reduced stability of their nucleoprotein or reverse transcription complexes (18, 46, 55) and are impaired in the reverse transcription of their genomes (13, 43, 55, 61, 66). Alternatively, it is possible that Vif, due to its ability to associate with viral nucleoprotein complexes as well as the cytoskeleton (31, 34), functions as an adapter to link the viral nucleoprotein or preintegration complex to a cellular transport pathway to facilitate its transport to the nuclear membrane. Such nuclear targeting mechanisms have been reported for other viruses, including herpes simplex virus 1 (59), human foamy virus (50), and adenovirus (64). For these viruses, incoming capsids are targeted to the nucleus in a microtubule-dependent mechanism. Interestingly, similar microtubule-dependent transport was recently observed for HIV-1 cores using green fluorescent protein-tagged HIV particles (T. Hope, personal communication). In addition, we have found that cells undergo a rapid change in their cytoskeletal organization immediately following infection by HIV-1, and we observed that the effect of Vif on the structure of vimentin is microtubule dependent (K. Strebel, unpublished observations). It is therefore possible that HIV, like other viruses, employs an active transport mechanism for nuclear targeting of its nucleoprotein complex. Although it is currently unclear if and how Vif would be involved in these events, it is conceivable that Vif functions to connect the viral core to a cytoskeleton-dependent cellular transport mechanism. Both models, i.e., the possible function of Vif in stabilizing viral cores and its proposed function in nuclear targeting of viral cores, would require only small amounts of Vif molecules but would depend on the presence of Vif in virions.

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